PHOTOENTRAINMENT OF CIRCADIAN RHYTHMS.

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A circadian clock is of no uese unless biological time is adjusted to local environmental time, andmost organisms use the changes in the quantity and quality of light at twilight as their primary zeitgeber to effect photoentrainment. The sensory demands of photoentrainment have imposed a unique set of selection pressures, which has led to the evolution of specialised photoreceptor systems. In the non-mammalian vertebrates, pineal and deep brain photorecceptors play an important, but poorly defined, role in circadian organisation. By contrast, photoentrainment in the mammals relies exclusively upon ocular photoreceptors. Although superficially very different, the entraining photoreceptor inputs of mammals and non-mammals appear to b both specialised (employing novel photopigments), and complex (utilising multiple photopigments). Why there should be this mulitplicity of photic inputs to the circadian system remains unclear, but must surely be related to the sensory task of twilight detection. During twilight, the quality of light changes in three important respects: (1) the amount of light; (2) the spectral composition of light; (3) and the position of the sum. In theory all of these parameters could be used by the circadian system to detect the phase of twilight, but each would be subject to considerable variation or "noise". Furthermore, the impact of this noise will depend upon the organism and the environment in which it inhabits. Thus the task of twilight detection is likely to be very complex and show considerable variation between species. If we are to place the molecular dissections of the circadian system into a functional context, then the ecology of photoentrainment must be given serious consideration.

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Summary: A circadian clock is of no use unless biological time is adjusted to local environmental time, and most organisms use the changes in the quantity and quality of light at twilight as their primary *zeitgeber* to effect photoentrainment. The sensory demands of photoentrainment have imposed a unique set of selection pressures, which has led to the evolution of specialised photoreceptor systems. In the non-mammalian vertebrates, pineal and deep brain photoreceptors play an important, but poorly defined, role in circadian organisation. By contrast, photoentrainment in the mammals relies exclusively upon ocular photoreceptors. Although superficially very different, the entraining photoreceptor inputs of mammals and non-mammals appear to be both specialised (employing novel photopigments), and complex (utilising multiple photopigments). Why there should be this multiplicity of photic inputs to the circadian system remains unclear, but must surly be related to the sensory task of twilight detection. During twilight, the quality of light changes in three important respects: (1) the amount of light; (2) the spectral composition of light; (3) and the position of the sun. In theory all of these parameters could be used by the circadian system to detect the phase of twilight, but each would be subject to considerable variation or "noise". Furthermore, the impact of this noise will depend upon the organism and the environment in which it inhabits. Thus the task of twilight detection is likely to be very complex and show considerable variation between species. If we are to place the molecular dissection of the circadian system into a functional context, then the ecology of photoentrainment must be given serious consideration.

1.0 Introduction.

The function of the circadian system is to co-ordinate the phase of a biological event to a specific feature of the 24-h environmental cycle, and to ensure that the phases of multiple rhythmic events within the organism are appropriately coupled. To achieve this timing, the circadian system must remain synchronised with the solar day. The entrainment of a biological clock requires an input pathway consisting of a receptor and transduction elements for the detection of specific environmental signals (*zeitgebers*). Furthermore, elements of the circadian clock must be capable of transforming the incoming signals to appropriate changes of the rhythm's phase. When entrained, the circadian clock adopts a distinct phase relationship with the astronomical day, and each of the expressed rhythms adopts its own phase relationships with the clock. Depending upon the species, biological clocks respond to a variety of different *zeitgeber*. For example, many microorganisms, plants, and heterothermic animals can be entrained by rhythmic changes in environmental temperature, whilst social signals such as feeding schedules in humans can act as *zeitgeber*. However, the stable daily change in the light environment at dawn or dusk provides the most reliable indicator of the time of the day. As a result, most organisms use the changes in the quantity and quality of light at twilight as their primary *zeitgeber* to effect photoentrainment.

2.0 Photoentrainment in Non-Mammals.

Non-mammalian vertebrates possess a diverse complement of photoreceptors, with several different types of photoreceptor organ developing from the embryonic forebrain (telencephalon). These photoreceptor organs can be classified as: (1) an intracranial pineal organ or pineal body (*epiphysis cerebri*) which is photoreceptive in all non-mammalian vertebrates; (2) an intracranial parapineal organ, found in many teleost fish and some agnatha; (3) an extracranial 'third eye', variously called a frontal organ (anura) or parietal eye/body (lacertids); (4) deep brain photoreceptors, which are located in several sites of the brain and are found in all non-mammalian vertebrates; and (5) lateral eyes, which contain photoreceptors in all the vertebrate classes. Of these extraretinal photoreceptors, the deep brain and pineal photoreceptors have been shown to play a critical role in the regulation of temporal physiology in the non-mammalian vertebrates. Very recent work in zebra fish has suggested that circadian rhythms of gene expression can be entrained by light in isolated organs (heart and kidney) (Whitmore *et al.*, 2000). However, critical experiments using light as a physiological stimulus need to be undertaken to distinguish between the truly photic and merely energetic/thermal effects of light on the effects observed.

2.1 Extraretinal Photoreceptors. Although highly variable in form, the vertebrate pineal has been shown to act as a photoreceptor in all non-mammalian species examined (Meissl and Yanez, 1994). Illumination of the non-mammalian pineal *in vitro* can modify melatonin synthesis in several different ways: (a) By entraining a circadian rhythm of melatonin synthesis (for example the lizard *Anolis*, see (Menaker and Wisner, 1983); (b) By regulating melatonin synthesis acutely (that is, melatonin synthesis is driven by a light:dark cycle and there is no endogenous clock driven rhythm in melatonin synthesis under constant conditions. For example the trout, see (Max and Menaker, 1992); (c) By regulating melatonin synthesis both acutely and by entraining a circadian rhythm of melatonin synthesis (for example the chicken, see (Takahashi *et al.*, 1989). Whether clock-driven, light-driven, or both, melatonin synthesis and release is confined to the dark portion of a light:dark cycle. The extent to which the melatonin signal is use by the circadian system varies greatly, both within and between the vertebrate classes. For example, in some birds

species (such as sparrows) the rhythmic release of pineal melatonin is essential for sustained rhythms in circadian behaviour under constant conditions (Gaston and Menaker, 1968), and infusion of melatonin can entrain circadian behaviour (for example in pigeons, (Chabot and Menaker, 1992). By contrast, pinealectomy has no obvious effects upon the circadian behaviour of other birds (for example Chicken and quail, (Menaker *et al.*, 1981). Why the pineal should have such differing roles in such closely related species remains a fascinating issue (Menaker and Tosini, 1996). Gwinner has proposed that the role of the pineal in avian circadian organisation is related to whether the species shows a long-distance or local migration each year (Gwinner *et al.*, 1997).

Deep brain photoreceptors were first linked to circadian entrainment as a result of the experiments undertaken by Menaker and colleagues during the 1960s and early 1970s. Studies on the house sparrow (*Passer domesticus*) showed that the removal of both eyes and pineal did not block photoentrainment (Menaker and Underwood, 1976). This observation was subsequently duplicated in many species of bird, fish, amphibia and reptiles (for review see (Foster *et al.*, 1993). It is important to note that neural tissue is remarkably permeable to light, and although light is scattered and filtered, photons penetrate deep into the brain (Foster and Follett, 1985). Although this light cannot be used to generate an image of the world, it can be used to calculate the overall amount of environmental light, and hence time of day. Deep brain photoreceptors have also been shown to play an important role in the regulation of the photoperiodic response of non-mammalian vertebrates. In the 1930s Benoit showed that blinded ducks exposed to spring-like photoperiods would be stimulated to breed (Benoit, 1935a; Benoit, 1935b). More recent studies, involving blinding and shielding light from entering the brain, have confirmed these original findings in a range of bird species (for review see (Foster and Follett, 1985). The pineal organ was originally assumed to regulate the avian reproductive responses, but it is now known that the pineal plays little or no role in the photoperiodic response. In quail, for example, the specific long-day illumination of the pineal does not stimulate gonadal growth (Homma *et al.*, 1980), and pinealectomy of either blind or eye intact quail leaves the photoperiodic response unaffected (Simpson *et al.*, 1983). By contrast, local illumination of the basal brain using fine fibre optics in blinded and pinealectomised quail, causes gonadal growth at normal rates (Oliver *et al.*, 1979; Yokoyama *et al.*, 1978).

2.2 The characterisation of extraretinal photoreceptors. Surgical lesions, directed illumination and *in vitro* analysis have demonstrated that the pineal (and other regions of the brain) contain photoreceptors, but characterisation of these photoreceptors has proved difficult. Describing the spectral sensitivity profile (action spectrum) of a light-dependent response is a critical step in identifying the regulatory photopigment of any system. The absorption spectrum of a photopigment gives a relative probability of photons being absorbed as a function of wavelength. As a result, the absorption spectrum of the photopigment dictates the spectral response of the photoreceptor. Accounting for any confounding factors such as screening pigments, the action spectrum of a light-response must match the absorbance spectrum of the photopigment/s upon which it relies. For example, the rod and cone photopigments of the vertebrates consist of an opsin protein coupled to a chromophore derived from an 11-*cis* form of vitamin A retinaldehyde. Despite great variation in their spectral maxima, rod and cone photopigments have spectra that match the standard template for a vitamin-A based photopigment ("Dartnall nomogram"). Significantly. action spectra for the light suppression of melatonin in many non-mammals (Deguchi, 1981; Meissl and Yanez, 1994), and an action spectrum for the deep brain photoreceptors mediating the photoperiodic response of quail (Foster *et al.*, 1985), also fit a Dartnall nomogram. Thus opsin-retinaldehyde

based photopigments were implicated in mediating these responses, and as a result, opsin and 11-*cis* retinaldehyde were sought within the CNS.

Rod- and cone-opsin specific antibodies have been found to label pinealocytes in a great variety of vertebrates, suggesting that both rod-like and cone-like photopigments are located within the pineal (Vigh and Vigh-Teichmann, 1988). However, in several studies, sub-sets of pinealocytes remained unlabelled by any opsin antibodies (Foster *et al.*, 1987). This failure was largely attributed to problems associated with either tissue fixation or antigenic sensitivities. The alternative possibility, that the pineal contained photopigments different from the rod and cone opsins of the eye, was given some consideration, but not demonstrated until the mid 1990s (see 2.3 below). A functional photopigment requires a chromophore and, in the limited number of studies undertaken, 11-*cis* retinoid has always been identified within the pineal of non-mammals (Foster *et al.*, 1989; Tabata *et al.*, 1985).

In contrast to the pineal, the localisation of opsins within the basal brain has proved much more of a problem. Many studies over a period of fifteen years, using a range of different anti-opsin antibodies, failed to give any clear localisation (Foster *et al.*, 1987; Vigh *et al.*, 1980; Vigh and Vigh-Teichmann, 1988). At the time it seemed inexplicable that anti-opsin antibodies that labelled pinealocytes within the avian pineal gland would fail to label any cells within the brain. This failure caused many researchers to dismiss the whole notion of encephalic photoreceptors. However, in 1988 Rae Silver's laboratory demonstrated that cerebrospinal fluid (CSF)-contacting neurones within the septal and tuberal areas of the brain of the ring dove, quail and duck could be labelled with an anti-rod opsin antibody. The impact of this finding was blunted because Western blots of these brain regions failed to validate the antibodies used (Silver *et al.*, 1988). Several years later, three new anti-cone opsin antibodies produced an intense immunostaining of CSF-contacting cells within the septal area of the brain of a lizard (*Anolis carolinensis*). Significantly, Western blots recognised a single 40 kD protein in ocular, anterior brain and pineal extracts, suggesting that the immunostaining observed was specific (Foster *et al.*, 1993; Grace *et al.*, 1993). Furthermore, the isolation of 11-*cis* retinoid from the *Anolis* forebrain, suggested that these opsins were functional (Foster *et al.*, 1993). Since 1993, several papers have considered opsin localisation within the central nervous system of a range of different vertebrates. For example, opsin-like labelling was found within the neurosecretory cells of the NMPO (nucleus magnocellularis preopticus) of the hypothalamus of several fish (Foster *et al.*, 1994) and amphibia (Yoshikawa *et al.*, 1994), and cells within the subhabenular of fish showed opsin labelling (Ekström *et al.*, 1987). Studies on the adult lamprey identified multiple populations of opsin immunoreactive CSF-contacting neurones throughout the hypothalamus, and non-CSF-contacting neurones were labelled in the epithalamic and caudal diencephalon (Garcia-Fernandez *et al.*, 1997).

Collectively, these results have implicated a number of different encephalic populations of photoreceptors: the pinealocytes of the pineal, CSF-contacting neurones adjacent to the third and lateral ventricles, non-CSF contacting cells of the NMPO, and other regions of the forebrain. Furthermore, these receptors utilise rod- and cone-like opsins and have additional photopigments that differ from both. The immunocytochemical studies suggested that extraretinal photoreception might be complex, and recent molecular findings have confirmed and extended this view.

2.3 Novel extraretinal photopigments. A key breakthrough in our understanding of the extraretinal photoreceptors has come with the application of molecular approaches to isolate novel extraocular opsins. The first of these opsins was isolated in 1994 by Fukada and colleagues who isolated a cDNA from the chicken pineal that encodes a photopigment with an absorption maximum near 470 nm (Okano *et al.*, 1994). This opsin was called 'pinopsin', and orthologues of pinopsin

have subsequently been isolated from the pineal of several different birds (Kawamura and Yokoyama, 1996) and lizards (Kawamura and Yokoyama, 1997). Pinopsin expression appears restricted to the pineal in birds, but may be expressed in both the pineal and retina of some reptiles. In 1997, the sequence of a novel fish opsin was described, VA (*vertebrate ancient*) opsin. VA opsin was originally isolated from Atlantic salmon (Soni and Foster, 1997), and more recently from carp (Moutsaki *et al.*, 2000). Salmon VA opsin forms a photopigment with an absorption maxima at 451 nm (with vitamin A_1) and is expressed in a sub-set of horizontal and amacrine cells of the retina, pineal, and subhabenular region of the brain (Soni *et al.*, 1998) (Philp *et al.*, 2000b). These findings have recently been duplicated in the zebrafish (Kojima *et al.*, 2000). Clearly, these results have important implications to both retinal and extraretinal photoreception. Another novel fish opsin, parapineal opsin, was isolated from catfish. This opsin is expressed primarily within the parapineal organ, but is also weakly expressed in the pineal (Blackshaw and Snyder, 1997). The functional properties of this opsin have yet to be determined. The most recent extraretinal opsin to be discovered is melanopsin (Provencio *et al.*, 1998c). Melanopsin was originally isolated from photosensory melanophores of *Xenopus laevis* but is also expressed in the hypothalamus (NMPO region), iris (photosensory in amphibia), and the horizontal cell layer of the retina (similar to VA opsin). Furthermore, an ortholog of *Xenopus* melanopsin has been isolated from chickens (Provencio *et al.*, 1998d) and fish (Rollag & Provencio - personal communication). Unfortunately expression studies are lacking, and we do not know whether melanopsin is capable of forming a functional photopigment in any of these vertebrates.

In addition to the discovery of novel opsins, recent molecular studies have suggested that there may be forms of rod and cone opsin that are unique to the extraretinal photoreceptors. For example, in several species of teleost fish, opsin cDNAs were isolated which share approximately 75% nucleotide and amino acid identity with their corresponding rod-opsins from the retina (Mano *et al.*, 1999; Philp *et al.*, 2000a). The basis for the sequence differences between the "extraretinal rod-like" (ERrod)-like opsins and retinal rod-opsins remains unclear, but may be related to the differing photosensory roles of the retinal and extra-retinal photoreceptors, and/or as a result of genetic drift of these opsins after a gene duplication event. Functional analysis of the ERrod-like photopigments, and the isolation of additional extra-retinal opsins should help resolve these alternatives. An additional example of pineal specialisation is the recent discovery of retinal and pineal specific arylalkylamine N-acetyltransferase (AANAT) genes isolated from two teleost fish - trout and pike. The evolution of two AANAT genes may represent a strategy for tissue optimisation of the photic regulation of melatonin synthesis (Coon *et al.*, 1999). This suggests that a number of the photosensory elements of the vertebrate pineal might be specialised for encephalic light detection.

2.4 Multiple extraretinal photopigments. Evidence for "encephalic photoreceptors" was first demonstrated in fish by Karl von Frisch in 1911. He showed that light-induced colour changes in the skin of the European minnow (*Phoxinus laevis*) would still occur in the absence of the eyes and pineal complex, and that lesions within the basal brain would block this response to light. von Frisch concluded that there must be photoreceptive elements within the diencephalon of fish (Frisch, 1911). Ninety years later we have evidence for not one, but multiple populations of photoreceptors within the diencephalon of fish and other vertebrates. Furthermore, the pineal complex of non-mammals appears to possess both novel and classical photopigments (Okano *et al.*, 1994; Philp *et al.*, 2000b). The future challenge will be to place these different photoreceptors into a physiological and behavioural context, and link the various opsins to a circadian or photoperiodic

response in the whole animal.

3.0 Photoentrainment in Mammals.

Until recently, all of the experimental evidence suggested that the circadian system of mammals is entrained by photoreceptors within the eye (e.g. (Nelson and Zucker, 1981). However, a recent paper suggested that bright light applied to the skin behind the knee (popliteal illumination) of humans could shift circadian rhythms of body temperature and rhythms of the hormone melatonin (Campbell and Murphy, 1998). These results were widely reported in the press but remain highly controversial. No instances of circadian entrainment in enucleated humans have been reported (Lockley *et al.*, 1997), and popliteal illumination produces no effect whatsoever on the suppression of nocturnal levels of pineal melatonin (Lockley *et al.*, 1998). Furthermore, illuminating the skin of blinded and shaved hamsters did not induce any phase shift (Yamazaki *et al.*, 1999). Unless and until other laboratories show that popliteal illumination can shift the human clock this form of extraocular photoreception in humans remains unproven.

Why the mammals have lost extraocular photoreceptors remains speculative, but has been correlated with the early evolutionary history of eutherian mammals and their passage through a nocturnal bottleneck (Foster and Menaker, 1993). Now although the entraining photoreceptors are ocular, the response characteristics of the circadian and visual systems to light are markedly different. In those mammals studied, the threshold for phase shifting is significantly higher than that required to elicit a visual response. For example, hamsters can recognise optical gratings at a luminance level 200 times less than the level necessary to elicit phase shifts in locomotor rhythms (Emerson, 1980). In addition, the circadian system appears markedly insensitive to light stimuli of a short duration. Indeed, the circadian system of the hamster is relatively insensitive to a stimulus duration of less than 30 seconds (Nelson and Takahashi, 1991). These features of the circadian system have been associated with filtering out those light stimuli, such as moon light and lightning, that would not provide information about the time of day (Roenneberg and Foster, 1997)

In mammals light information from the eye reaches the primary circadian oscillator in the suprachiasmatic nuclei (SCN) via a specific neural projection called the retino-hypothalamic tract (RHT). This tract arises from a small sub-set of retinal ganglion cells and forms a relatively small number of the fibres of the optic nerve. For example, in the mouse retina, only 0.1% of the RGCs form the RHT projection to the SCN (Provencio *et al.*, 1998a). Although the RGCs that form the RHT have been identified, the photoreceptors that are connected to these cells have not.

3.1 Novel Ocular Photoreceptors. Disentangling which retinal cells mediate photoentrainment from the mass of neurones dedicated to image detection has been a major problem. The retina of all mammals contains two types of known photoreceptors: rods which are typically associated with dim light vision, and cones which are associated with colour vision under bright light conditions. Trying to establish whether these photoreceptors mediate the effects of light on the biological clock has been a complex issue. However, the use of mice with naturally occurring genetic disorders of the eye has provided a partial solution to this issue. Mice homozygous for *retinal degeneration* (*rd/rd*) were the first animals to be used for such studies. Despite the massive loss of rods and cones, *rd/rd* mice show circadian responses to light that are unattenuated when compared with non-degenerate control mice (*rd/+*, *+/+*). Thus the sensitivity of the circadian system to light does not parallel loss of either rod or cone photoreceptors (Provencio *et al.*, 1994). However, removal of the eyes abolishes all circadian responses to light in *rd/rd* mice (Foster *et al.*, 1991). The remarkable findings in *rd/rd* mice showed that rods are not required

for photoentrainment, but the eyes must contain these light sensing cells. In more recent studies in humans, a substantial portion of patients who have eyes but had lost their vision due to retinal disease, also retained their ability to suppress melatonin (Czeisler *et al.*, 1995) as well as to shift their circadian phase (Lockley *et al.*, 1997).

The most recent experiments to address the impact of rod and cone photoreceptor loss on the circadian system employed mouse models that lacked all rods and cones. In rodless+coneless mice, circadian responses to light (photoentrainment and pineal melatonin suppression) were intact (Freedman *et al.*, 1999; Lucas *et al.*, 1999). These results lead to the striking conclusion that mammals must use some unidentified photoreceptor outside the rod and cone receptors. Again, removal of the eyes abolished all circadian responses to light demonstrating that the eyes must house these unrecognised photoreceptors (Freedman *et al.*, 1999).

3.2 A novel opsin photopigment in the mammalian retina. Although the requirement of rod and cone opsins in circadian light reception has been excluded, there is evidence for involvement of an opsin-based pigment in this response. In rodents two detailed action spectra for photoentrainment have been undertaken. In both the golden hamster (Takahashi *et al.*, 1984) and mouse (Provencio and Foster, 1995), the data showed a very close fit to a standard template for a vitamin-A based photopigment (see 2.2). These findings, taken together with the results from rodless+coneless mice, suggest that the mammalian eye contains unrecognised non-rod, non-cone opsin-based photoreceptors within the inner retina which regulate circadian rhythms. The conclusion that the vertebrate inner retina contains novel photoreceptors is given direct support by the discovery of VA photopigments within the inner retina of fish (Soni *et al.*, 1998) (see 2.3). To date, no VA opsin homologs have been identified in mammals, but a number of other candidates exist. These include RGR (retinal-binding G protein-coupled receptor), peropsin, and encephalopsin, and melanopsin. With the exception of encephalopsin (Blackshaw and Snyder, 1999), which is expressed in a variety of neural and non-neural tissues, and therefore falls outside of the scope of this review, they are all ocular. RGR, differs from rod and cone opsins in that its preferred chromophore is all-*trans*-retinaldehyde rather than 11-*cis*-retinal. Upon exposure to light, RGR photoisomerises the all-*trans* chromophore to the 11-*cis* confirguration. Its likely function, therefore, is not that of a signalling photopigment, but rather that of a photoisomerase (Hao and Fong, 1999). Furthermore, its localisation to the retinal pigment epithelium (RPE) and Müller cells (Jiang *et al.*, 1993) casts doubts as to whether it could initiate a signal that would directly or indirectly communicate through the ganglion cells of the retinohypothalamic tract to the SCN. Peropsin, is also localised to the RPE. The role of peropsin in unknown, but several lines of evidence suggest that it may function like RGR (Sun *et al.*, 1997a). The most recent, and arguably the best candidate to date, is a mammalian homolog of *Xenopus* melanopsin (see 2.3) which differs from all of the other novel mammalian opsins in that it is expressed within the neural retina (Provencio *et al.*, 2000). Specifically, melanopsin is expressed in a small number of cells within the ganglion and amacrine cell layers in the inner retina. This distribution is strikingly similar to the distribution of murine retinal ganglion and amacrine-like cells known to form the retinohypothalamic tract (Provencio *et al.*, 1998b). Once again, functional expression studies are lacking, and we do not know whether this mammalian form of melanopsin is capable of forming a functional photopigment.

It is worth noting that in addition to circadian physiology, many other aspects of mammalian biology are influenced by gross changes in environmental light, including pupil size, blood pressure, mood and attention (Wetterberg, 1993). It is possible, therefore, that uncharacterised ocular photoreceptors might form the basis

of a general "irradiance detection" pathway mediating many, if not all, non-visual responses to light. Support for this hypothesis comes from our recent work on rodless+conless mice. In addition to circadian responses to light, these animals also show a pupillary light reflex. The action spectrum for this response demonstrates the involvement of an opsin/vitamin A-based photopigment with a wavelength of maximum sensitivity at 479 nm. The known mouse photopigments have maximal absorbances at wavelengths of 360 nm (UV cones, (Jacobs *et al.*, 1991), 498 nm (rods, (Bridges, 1959) and 508 nm (Green cones, (Sun *et al.*, 1997b), and so cannot account for the pupillary responses observed in rodless+coneless mice (Lucas *et al.*, 2000). Until, we have matched the action spectra for pupillary and circadian responses to light it remains possible that these aspects of physiology are driven by different novel photoreceptors. However, the principle of parsimony would argue against this, and directs the search for circadian photopigment to those candidates that conform to the basic opsin:11-cis retinaldehyde composition and, in mice, have spectral maxima near 479nm.

In addition to opsin-based photopigments, recent studies in the plant *Arabidopsis*, the fruit-fly *Drosophila* and mice suggested that a group of proteins, called the cryptochromes (*cry*), might be responsible for detecting light and mediating photoentrainment in phylogenetically diverse organisms (Devlin and Kay, 1999). In mammals at least, the evidence for this hypothesis was always weak (Lucas and Foster, 1999a; Lucas and Foster, 1999b), and has recently collapsed as a result of detailed studies by Griffin *et al* (Griffin *et al.*, 1999) who failed to uncover any effect of light on the activity of these proteins in cell culture. Furthermore, the disruption of the cryptochrome genes (CRY1 and CRY2) does not block the light-induced expression of two "clock genes" in the SCN of mice (Okamura *et al.*, 1999).

3.3 A role for rods and cones. There is overwhelming evidence that unidentified (non-rod, non-cone) photoreceptors within the mammalian eye mediate photoentrainment. However, this does not mean that the classical rod and cone photoreceptors play no role in this process. The experiments on rodless+coneless mice outlined above merely suggest that these receptors are not required. Indeed, indirect evidence for the involvement of cone photoreceptors in photoentrainment comes from studies on an extraordinary animal - the blind mole rat (*Spalax ehrenbergi*). *Spalax* is a subterranean fossorial rodent with subcutaneous atrophied eyes and shows a massive reduction (87-97%) of those regions of the brain associated with the image-forming visual system (Cooper *et al.*, 1993). Although visually blind (Haim *et al.*, 1983; Rado *et al.*, 1992), the minute eyes (little more than 0.5 mm in diameter) can perceive light and are used to entrain circadian rhythms (David-Gray *et al.*, 1998; Goldman *et al.*, 1997; Rado *et al.*, 1991). Photoentrainment is thought to occur in the wild when *Spalax* removes debris from the tunnel complex and is exposed to brief periods of natural light (Rado, 1989). Over the past 30 million years, evolutionary processes appear to have disentangled and eliminated the image-forming visual system of this animal whilst retaining those components of the eye that regulate the biological clock. Remarkably, a cone opsin has been isolated from the eye of *Spalax*, and this opsin has been shown to form a fully functional photopigment (David-Gray *et al.*, 1998). These results provide strong, although indirect, evidence that cone photopigments contribute to photoentrainment in *Spalax*, and by implication, other mammals.

4.0 Multiple photopigments and twilight detection

The conclusion, based upon studies in *Spalax*, that cone photopigments might contribute to photoentrainment in all mammals (see 3.3) would appear to contradict the findings that the loss of both rod and cone photoreceptors has no effect on rodent photoentrainment, and that the retina contains novel circadian

photoreceptors (Freedman *et al.*, 1999; Lucas *et al.*, 1999). These results appear less contradictory, however, when the pineal organs of non-mammals are considered. As discussed above, in non-mammalian vertebrates the pineal organ may be both an important part of the circadian timing system (in some ways analogous to the mammalian SCN) and is itself directly light sensitive. This photosensitivity is attained using multiple photopigments, with rod- and cone-like opsins, as well as novel opsins, co-existing within the same organ (Shand and Foster, 1999). Thus both novel and classical photopigments seem to mediate the effects of light on temporal physiology in all the vertebrate classes. And the question is why? If this question is to be addressed, then we must first define those features of the light environment, which are important for the regulation of temporal physiology.

During twilight, the quality of light changes in three important respects: 1) the amount of light, 2) the spectral composition of light, 3) and the source of light (i.e. the position of the sun). These photic parameters all change in a systematic way, and in theory all could be used by the circadian system to detect the phase of twilight (Roenneberg and Foster, 1997). However, each of these parameters is subject to considerable sensory "noise" (Table 1), and the impact of this noise will depend upon the organism and the environment in which it inhabits. One can also make the general point that in all sensory systems, much of the complexity observed is associated with noise reduction. A classic example of this in the visual system in colour vision. Colour vision is a mechanism for increasing the signal to noise of an object against its background by exploiting the fact that different objects do not reflect the same wavelengths of light equally.

Table 1.

(1) Variation in the stimulus	
Channel/Signal Noise	Fluctuations in the light signal, e.g. cloud cover or daylength.
Environmental Noise	Extraneous signals from other sources of light, e.g. starlight, moonlight, and lightning.
Receptor Noise	Molecular noise of the receptor pathway, e.g. variation in external temperature.
(2) Variation in stimulus exposure	
Sensory Adaptation	Changing receptor thresholds, e.g. receptor habituation, changes in pupil size.
Behavioural Noise	Behavioural state, e.g. emergence from burrow, feeding, courting etc., etc.
Developmental Noise	Stage of development, e.g. feeding niche, body pigmentation, neural connections, developmental niche (egg or in uterus) etc.

Table 1. *The major sources of "noise" associated with the photic regulation of temporal physiology. Like other sensory systems (Dusenbery, 1992), the two main sources of noise for twilight detection are associated with variation in the light stimulus and variation in exposure to the light stimulus. In each case the impact of this noise will depend upon the organism and the environment in which it inhabits. Some examples of the possible types of noise that might be expected to complicate photoentrainment are listed.*

5.0 The Future: Until recently circadian biologists have tended to use light merely as a "hammer" to shift the clock. Our work has shown that twilight detection is not a straightforward stimulus. It is highly dynamic and subject to considerable noise. On

the basis of what we know about other sensory systems we would predict that the photic inputs regulating temporal physiology will be both specialised and complex. Indeed, we have demonstrated the presence of both novel (specialised) and multiple (complex) inputs regulating temporal physiology in mammals and non-mammalian vertebrate classes. Furthermore, multiple photopigments appear to contribute to photoentrainment in organisms as diverse as *Gonyaulax* (Roenneberg and Deng, 1997) and *Drosophila* (Stanewsky *et al.*, 1998). In view of the considerable progress that has been made in associating different photoreceptors to the circadian system, it is now time for circadian biologists to stop asking "what is the circadian photopigment" and ask the more sophisticated question "how do multiple photic channels interact to reduce the noise problem inherent in twilight detection."

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